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BRIEF REPORT

Glucagon-like peptide-1 receptor expression in the human eye

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Semaglutide is a human glucagon-like peptide-1 (GLP-1) analogue that is in development for the treatment of type 2 diabetes. In the pre-approval cardiovascular outcomes trial SUSTAIN 6, semaglutide was associated with a significant increase in the risk of diabetic retinopathy (DR) complications vs placebo. GLP-1 receptor (GLP-1R) expression has previously been demonstrated in the retina in animals and humans; however, antibodies used to detect expression have been documented to be non-specific and fail to detect the GLP-1R using immunohistochemistry (IHC), a problem common for many G-protein coupled receptors. Using a validated GLP-1R antibody for IHC and in situ hybridization for *GLP-1R* mRNA in normal human eyes, GLP-1Rs were detected in a small fraction of neurons in the ganglion cell layer. In advanced stages of DR, GLP-1R expression was not detected at the protein or mRNA level. Specifically, no GLP-1R expression was found in the eyes of people with long-standing proliferative DR (PDR). In conclusion, GLP-1R expression is low in normal human eyes and was not detected in eyes exhibiting advanced stages of PDR.

KEYWORDS

cardiovascular disease, diabetes complications, diabetic retinopathy, GLP-1 analogue

1 | INTRODUCTION

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) provide benefits for people with type 2 diabetes (T2D), including improvements in glycaemic control and body weight, and two GLP-1RAs have also shown cardiovascular (CV) risk reduction. The efficacy and safety of semaglutide in patients with T2D were assessed in the SUSTAIN clinical trial programme, in which semaglutide provided superior improvements in glycated haemoglobin (HbA1c) and body weight vs comparators, and furthermore showed CV risk reduction in SUSTAIN 6, a 2-year pre-approval CV outcomes trial.[1,2] In SUSTAIN 6, semaglutide was associated with a higher rate of diabetic retinopathy (DR) complications.[2] DR complications were an adjudicated composite secondary endpoint, based on time from randomization to the first occurrence of need for retinal photocoagulation or treatment with intravitreal agents, diagnosis of vitreous haemorrhage, or onset of diabetes-related blindness. Unlike most phase III T2D trials, in SUSTAIN 6, patients with advanced diabetes and high risk of CV disease were included, with no upper limit on HbA1c.[2] A recently published paper from SUSTAIN 6 concludes that early worsening of DR is a known phenomenon associated with the rapidity and magnitude of improvement in glycaemic control with insulin, and the DR findings in SUSTAIN 6 are consistent with this.[3]

The glucagon-like peptide-1 receptor (GLP-1R) is a G-protein coupled receptor (GPCR) that shows high homology among species. Detection of GPCRs by immunohistochemistry (IHC) is technically difficult and many antibodies have been shown to be unspecific.[4,5] As a result, journals have introduced rigorous reporting requirements for IHC results.[6] When reporting cellular localizations for a GPCR it is important to use not only IHC, but also a complementary technology, such as in situ hybridization (ISH) or in situ ligand binding (ISLB). For cellular localization, ISLB is not optimal and ISH is preferred to complement IHC. Similarly to other GPCRs, data on GLP-1R expression with IHC has been subject to controversy because of non-specific antibodies.[7,8] Many published papers describe studies with polyclonal antibodies that have subsequently been withdrawn from the market because of a confirmed lack of specificity.[9] Specific monoclonal antibodies are available, and use of these in IHC, combined with ISLB, has provided thorough characterization of

GLP-1R expression in tissues of particular interest, such as the pancreas, kidney and thyroid.[10,11]

We have now investigated GLP-1R expression in normal human eyes and eyes of patients with advanced stages of proliferative DR (PDR) by IHC with the thoroughly validated monoclonal antibody 3F52. The IHC analysis was confirmed by the detection of *GLP-1R* mRNA by RNAScope ISH. This technique has become state-of-art for ultra-sensitive and specific mRNA detection.[12]

2 | METHODS

2.1 | Tissue samples

The human eye samples were formalin-fixed, paraffin-embedded sections from patients with PDR (n = 5, mean ±SD [range] age 47 ±12 [28-67] years; two men; all had a diabetes duration >10 years and all had received laser photocoagulation) and controls (n = 4, mean ±SD [range] age 62 ±8 [49-70] years; three men). All patients had enucleation carried out because of pain and had PDR according to the International Clinical Diabetic Retinopathy Severity Scale outlined by the American Academy of Ophthalmology.[13] The control subjects had eyes enucleated as a result of extraocular cancer treatment; eyes were clinically and histologically classified as normal, and no patients received radiotherapy. Human positive control tissue was provided by Asterand Bioscience (Royston, UK). The study was performed as a collaboration between the Department of Pathology at Rigshospitalet (Copenhagen, Denmark) and Novo Nordisk A/S and was approved by the Regional Committee on Health Research Ethics for the Capital Region of Denmark (H-15014782). Tissue from rhesus monkeys was used to optimize the protocol before use on the human samples. Sampling from rhesus monkeys was performed according to regulations specified under the Protection of Animals Act by the European Union authority. The tissues were paraformaldehyde-fixed and paraffin-embedded.

2.2 | Immunohistochemistry and immunofluorescence

Sections (3-5-μm thickness) were cut and antigen retrieval was performed in Tris-EGTA buffer (pH 9.0) at 99°C for 15 minutes. The slides were preincubated for 30 minutes in protein-blocking solution (FP1012; Perkin Elmer, Waltham, Massachusetts) and incubated overnight at 4°C with the primary antibodies diluted in the protein blocking solution. The primary antibodies were detected with BrightVision Poly horseradish peroxidase (HRP) anti-mouse IgG (DPVM55HRP; Immunologic) followed by Discovery Purple HRP (760-229; Roche, Basel, Switzerland). The antibodies used were mouse anti-GLP-1R (2.5 or 7.5 μg/mL, 3F52; Novo Nordisk A/S, Denmark) and mouse IgG1 isotype control (MAB002; R&D Systems, St Paul, Minneapolis). For immunofluorescence, the following additional antibodies were applied: rabbit anti-neuronal nuclei (NeuN [0.3 μg/mL, Ab177487; Abcam, Cambridge, UK]), rabbit anti-GFAP (0.2 μg/mL, Z0334; DAKO, Glostrup, Denmark); rabbit IgG isotype control (910801; BioLegend, San Diego, California); Alexa488-conjugated goat anti-rabbit IgG (2 μg/mL, A-11034; Thermo Fisher Scientific, Waltham, Massachusetts); and Alexa594-conjugated goat anti-mouse IgG (8 μg/mL, A-11032; Thermo Fisher Scientific). All NeuN positive cells in the ganglion cell layer (GCL) in one eye section per donor were counted in the GLP-1R IHC stains.

2.3 | In situ hybridization

Single and duplex ISH were performed with the RNAscope 2.5 VS Reagent Kit (322260; Advanced Cell Diagnostics, Newark, California) and RNAscope 2.5 LS Duplex Reagent Kit (322440; Advanced Cell Diagnostics), respectively. Pretreatment for single ISH was 8 minutes at 97°C and 12-minute protease treatment and, for duplex ISH, 10 minutes at 88°C and 10-minute protease treatment. The probes applied were all targeting human mRNA; that is, *GLP-1R* (519829, 519828), platelet endothelial cell adhesion molecule-1 (*PECAM1*; 548458), RNA polymerase II subunit A (*POLR2A*; 310459, 310458) and melanopsin (*OPN4*; 504998), all from Advanced Cell Diagnostics. Dihydrodipicolinate reductase (*DAPB*; 312039VS, 312038LS) codes for bacterial mRNA, and served as a negative control probe. For all experiments *GLP-1R* was detected by Fast Red-based kits; in duplex stain, *GLP-1R* was detected in combination with a green chromogen (322550; Advanced Cell Diagnostics).

All bright field pictures were obtained with a Hamamatsu Nanozoomer 2.0 HT slide scanner (pixels 1024 × 1024) and all fluorescent pictures were obtained with a Zeiss AXIO, Imager 2 epifluorescent microscope (pixels 1388 × 1040).

3 | RESULTS

3.1 | GLP-1R expression in normal human eyes

In normal human eyes (n = 4), GLP-1Rs were detected in single cells in the retinal GCL (Figure 1A). GLP-1Rs were not detected in the retinal and choroid vasculature, the retinal pigment epithelium, or in any other ocular structure. The GLP-1R-positive cells constituted <1% of the cells in the GCL, and only 0-4 GLP-1R-positive cells were detected per eye section (4/1547, 3/1598, 0/1448 and 2/1479 GLP-1R-positive neurons/ NeuN-positive neurons in the GCL). The GLP-1R-positive cells were morphologically characterized by having a round nucleus and horizontal processes. The detection of only a low number of GLP-1R-positive cells in the retina could not be explained by low sensitivity of the staining protocol because the positive control tissues, stained in parallel with the eye sections, showed the expected pattern of GLP-1R expression. Strong GLP-1R staining was detected in the Brunner's glands in the duodenum (human) and in the pancreatic islets (monkey). Weaker GLP-1R immunoreactivity was detected in acinar cells in the pancreas and at the vascular pole in the renal cortex (monkey; Figure 1C,E). *GLP-1R* mRNA was detected in single cells in the GCL (n = 4; Figure 1G). Staining with positive (*POLR2A*) and negative (*DAPB*) control probes showed a good signal-to-noise ratio with the applied protocol and sufficient mRNA integrity for performing ISH (Figure 1H,I). No *GLP-1R* mRNA was detected outside the GCL.

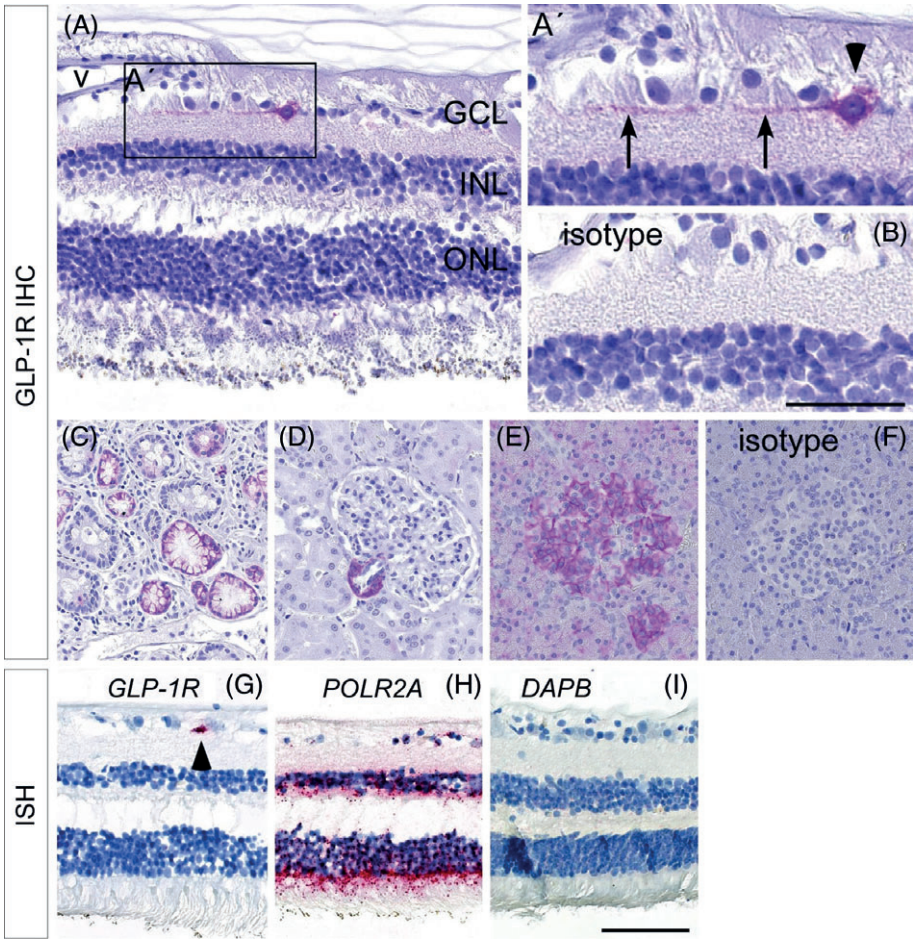

FIGURE 1 Detection of single glucagon-like peptide-1 receptor (GLP-1R)-positive cells in the human retina. A, detection of GLP-1R protein by immunohistochemistry (IHC) in single cells of the ganglion cell layer (GCL) in the human retina (pink colour). C–E, detection of GLP-1R protein in C, Brunner's glands and D, rhesus monkey kidney and E, pancreas, serving as positive control tissues. B, and F, no immunoreactivity was detected when the antibody against GLP-1R was substituted with an isotype control antibody, either in the eye (B) or in the control tissues (F). G, detection of *GLP-1R* mRNA by in situ hybridization (ISH) in single cells localized in the GCL. H and I, the positive control probe (*POLR2A*) (H) confirmed the preservation of mRNA in the sections and the negative bacterial control probe (*DAPB*) (I) showed the level of unspecific staining. The control probes showed a good signal-to-noise ratio. All sections were counterstained with haematoxylin. One eye section was stained per donor. Arrow heads, GLP-1R-positive cells; arrows, GLP-1R-positive process; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; v, blood vessel. Scale bars represent 50 μm (B) and 100 μm (I) and apply to A´ and B, and C–I, respectively

To characterize the GLP-1R-positive cells in the GCL, double immunofluorescence was performed. GLP-1Rs were detected in cells co-labelled with the neuronal marker NeuN (Figure S1A), but not in glial fibrillary acidic protein (GFAP)-positive glial cells (Figure S1B; $n = 3$). No co-labelling was detected when the primary antibodies were replaced with isotype control antibodies (data not shown). Based on the position and low frequency of the GLP-1R-positive cells, they could be melanopsin-expressing retinal ganglion cells. To address this, duplex ISH for *GLP-1R* and *OPN4* (coding for melanopsin) was performed. Only one out of 13 GLP-1R-positive cells co-localized with melanopsin ($n = 4$; Figure S1C).

3.2 | GLP-1R expression in PDR

The expression of GLP-1Rs was studied in eye sections from patients with advanced stages of PDR ($n = 5$). Different histopathological findings representative for DR, including neuronal cell loss, retinal pigment epithelium proliferation and areas of neovascularization, were present in the sections. The five donor sections had 287, 96, 326, 732 and 520 neurons, respectively, in the GCL. The GLP-1R protein could not be detected in the retina or any other ocular structure in sections from patients with PDR. Areas with neovascularization and no GLP-1R expression are shown in Figure 2A,B. Furthermore, *GLP-1R* mRNA was not detected in the PDR sections, although strong staining was obtained in the sections with positive control probes (Figure 2C,D).

4 | DISCUSSION

We investigated GLP-1R expression in human eyes with and without PDR. GLP-1Rs were detected in normal human eyes, with full agreement between the detection of GLP-1R protein and mRNA. GLP-1Rs were only detected in single cells confined to the GCL of the retina. No expression was detected in the retinal or choroidal vasculature or in the retinal pigment epithelium. Based on the morphology of the GLP-1R-positive cells and our cell-identification analysis, the GLP-1R-positive cells appeared to have a neuronal phenotype. Disruption of the circadian rhythm in patients with DR has been reported,[14] and melanopsin-expressing retinal ganglion cells are known for regulating the circadian rhythm.[15] The low frequency and position of the GLP-1R-positive cells suggest that these cells could represent melanopsin-expressing retinal ganglion cells[15]; however, we only detected a limited overlap between GLP-1R and melanopsin in the normal human eyes. A publication using db/db mice has indicated GLP-1RAs may confer neuroprotection in DR.[16] As animal models should always be interpreted with caution, it is likely that studies in other animal models reflecting other aspects of DR would be needed for a full understanding of the potential of GLP-1RAs in DR.[17] A DR outcome study in patients with type 2 diabetes will be conducted with semaglutide.

Neither GLP-1R protein nor mRNA was detected in eye sections from patients with advanced stages of PDR. Importantly, we did not detect any signs of GLP-1R expression in areas characterized by neovascularization. The lack of GLP-1R expression in the GCL may be

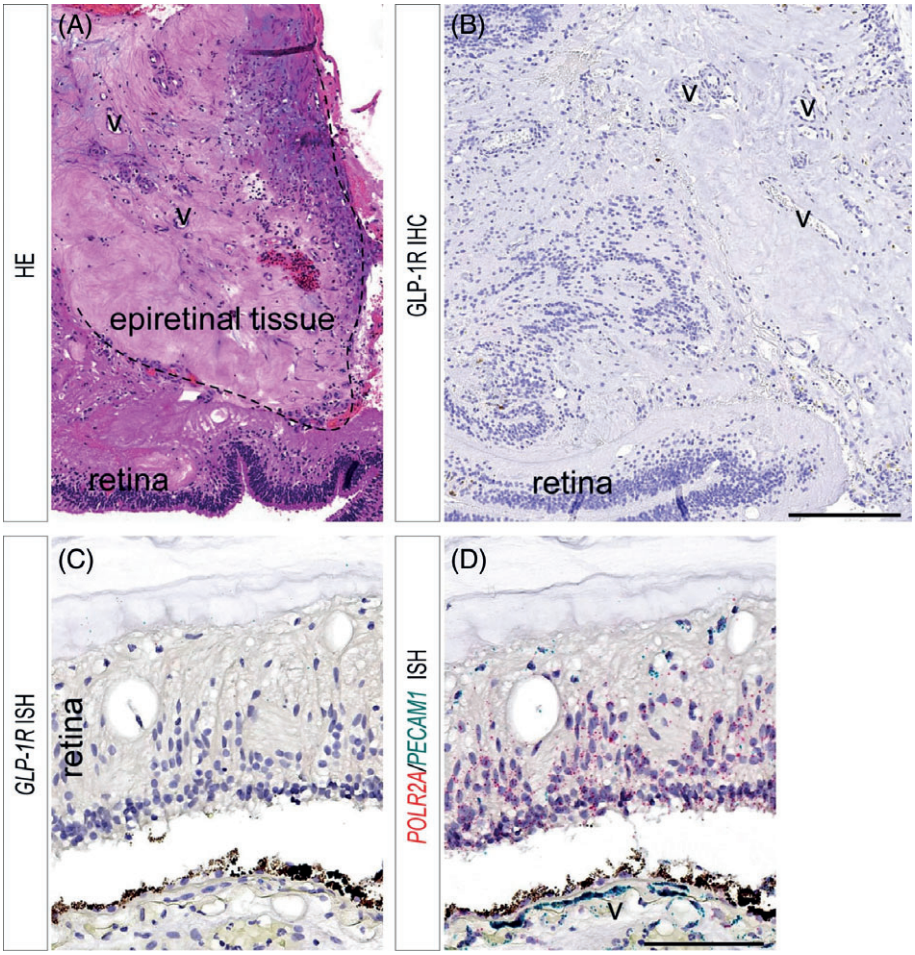

FIGURE 2 Glucagon-like peptide-1 receptor (GLP-1R) protein and mRNA were undetectable in proliferative diabetic retinopathy (PDR). A, Retina with epiretinal tissue with neovascularization from a patient with PDR. B, GLP-1R immunohistochemistry (IHC) from the same area as in A. GLP-1R was not detected in the retina or in areas of neovascularization. C, *GLP-1R* mRNA was likewise not detected in the eyes from patients with PDR. D, Positive controls [POLR2A (red) and PECAM1 (green)]. All sections were counterstained with haematoxylin. One eye section was stained per patient. HE, haematoxylin and eosin; v, blood vessel. Scale bars represent 250 μm (B) and 100 μm (D), respectively. Scale bar in B applies to A–B and scale bar in D to C–D

explained by partial loss of retinal ganglion cells at these stages of the disease.[18] Because of the expression pattern of GLP-1Rs detected in normal eyes, GLP-1Rs are likely to be present in the GCL at less advanced stages of the disease where the neuronal cell loss is less profound. To what extent GLP-1R expression is induced in other structures at earlier stages of DR or PDR was not addressed, but the present data do not suggest ectopic expression of GLP-1Rs in any ocular structure.

Other studies using rodent animal models reflecting early diabetic eye disease have suggested that GLP-1RAs could prevent the occurrence of DR, and show GLP-1R expression in the retina, in the GCL, inner nuclear layer and outer nuclear layer[16]; however, the GLP-1R expression methodology applied in those studies with IHC is not valid because they used antibody ab39072, which has been withdrawn from the market as a result of a confirmed lack of specificity.[9] With the limited GLP-1R expression and the confinement to neurons in GCL in normal human eyes it can be hypothesized that GLP-1RAs are unlikely to play a direct role via the GLP-1R in the worsening of established DR.

The present data are in contrast to the literature suggesting more abundant retinal expression of GLP-1Rs.[16] In the present study, we used IHC with a thoroughly validated GLP-1R antibody, the inclusion of negative control antibodies and positive control tissues. Furthermore, the IHC analysis was confirmed by the detection of *GLP-1R* mRNA by ISH.

A limitation of the study is the number of donors and eye sections, and having only advanced stages of PDR included. The PDR samples were from patients treated with photocoagulation; thus, the possibility that the treatment had an impact on the results cannot be fully excluded. The GLP-1R expression results were nevertheless consistent across the different analyses performed. These results highlight the need for caution when performing analyses of GLP-1R expression, as apparently positive findings may be "false-positive" as a result of lack of specificity of the analytical methods.

In conclusion, GLP-1Rs are rarely expressed in the normal human eye and appear undetectable in advanced stages of PDR.

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Author contributions

Author contributions were as follows. J.B.H.: study design and conduct and writing the manuscript; L.B.K., S.H., P.H.K.: data review and writing the manuscript; C.P.: data and protocol review; and E.Y.: clinical input. P.H.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest

S.H. declares no relevant conflict of interest. J.B.H. is a former employee of Novo Nordisk. C.P., E.Y., L.B.K. and P.H.K. are employees of Novo Nordisk. J.B.H., C.P., L.B.K. and P.H.K. are shareholders of Novo Nordisk.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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